Photochemical Mechanism of Light-Driven Fatty Acid Photodecarboxylase

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ABSTRACT: Fatty acid photodecarboxylase (FAP) is a promising target for the production of biofuels and fine chemicals. It contains a flavin adenine dinucleotide cofactor and catalyzes the blue-light-dependent decarboxylation of fatty acids to generate the corresponding alkane. However, little is known about the catalytic mechanism of FAP, or how light is used to drive enzymatic decarboxylation. Here, we have used a combination of time-resolved and cryogenic trapping UV−visible absorption spectroscopy to characterize a red-shifted flavin intermediate observed in the catalytic cycle of FAP. We show that this intermediate can form below the "glass transition" temperature of proteins, whereas the subsequent decay of the species proceeds only at higher temperatures, implying a role for protein motions in the decay

of the intermediate. Solvent isotope effect measurements, combined with analyses of selected site-directed variants of FAP, suggest that the formation of the red-shifted flavin species is directly coupled with hydrogen atom transfer from a nearby active site cysteine residue, yielding the final alkane product. Our study suggests that this cysteine residue forms a thiolate-flavin charge-transfer species, which is assigned as the red-shifted flavin intermediate. Taken together, our data provide insights into light-dependent decarboxylase mechanisms catalyzed by FAP and highlight important considerations in the (re)design of flavin-based photoenzymes.
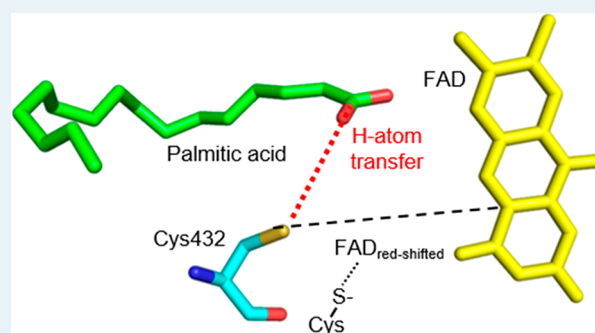
KEYWORDS: flavin, fatty acid photodecarboxylase, photoenzyme, red-shifted species, thiolate, charge transfer, decarboxylation

The recently discovered light-activated enzyme, fatty acid photodecarboxylase (FAP),[1] provides a highly attractive route to the production of alkanes and other chemicals of potential industrial importance.[2−8] FAP is found in unicellular photosynthetic green microalga and catalyzes the blue-light-driven decarboxylation of fatty acid substrates to generate the corresponding *n*-alkane/alkene product. The enzyme contains a flavin adenine dinucleotide (FAD) cofactor and has a preference for long-chain fatty acid substrates (C16−C17).[1] A number of recently published studies have demonstrated FAPs have the potential to be used for industrial biotechnological purposes. The substrate scope of FAP can be altered by protein engineering methods[4,8] and the use of decoy molecules.[3] FAP has been used in conjunction with additional enzymes to generate photoenzymatic cascades for the synthesis of long-chain aliphatic amines and esters.[7] Moreover, a mechanistic understanding of FAP photochemistry could be informative on the repurposing of thermally activated flavoenzymes as new photobiocatalysts.[9−11] In recent years, photoexcitation of flavin-dependent "ene" reductases has opened up new reaction channels, allowing these enzymes to catalyze ketone reduction[9] and asymmetric radical cyclization reactions.[10,11] These are exciting applications of flavoenzymes in photobiocatalysis. However, for further exploitation as next-generation photo-

biocatalysts, a deeper understanding of the reaction mechanism in both natural and repurposed, thermally activated flavoenzymes is required.

FAP is one of only three light-dependent enzymes found in nature, the others being DNA photolyase[12,13] and protochlorophyllide oxidoreductase.[14,15] As they do not require complex rapid mixing strategies to initiate and probe catalysis, light-dependent enzymes offer a number of experimental advantages compared to thermally activated enzyme systems. Specifically, the enzyme−substrate complex can be formed in the dark prior to catalysis, which provides a unique opportunity to initiate catalysis at cryogenic temperatures and with very fast laser pulses.[13−15] Therefore, the reaction chemistry of light-dependent enzymes can be monitored over a wide range of temperatures and on very short time scales, which are not generally accessible for the majority of enzymes.[12−15] Time-resolved spectroscopy has previously
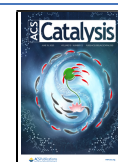
been used to support the proposal that FAP-catalyzed decarboxylation occurs on the picosecond−nanosecond time scale.[1] It was suggested that electron transfer from the fatty acid substrate to the photoexcited FAD occurs on the picosecond time scale, generating a highly reactive fatty acid radical (Figure 1). Formation of this radical facilitates
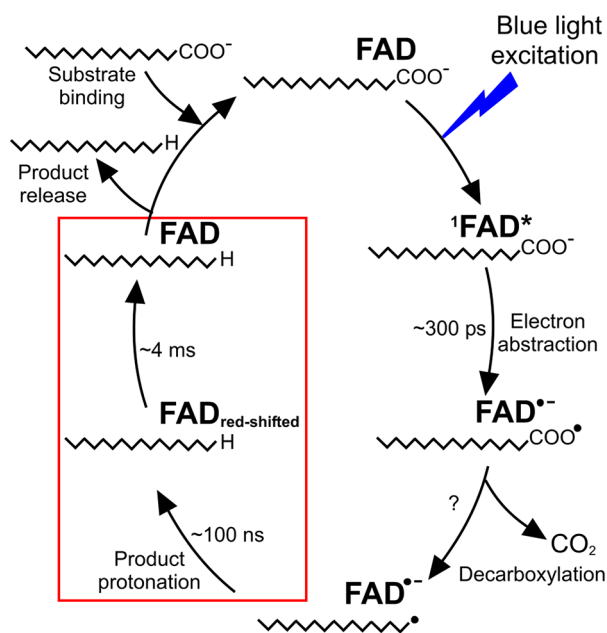

Figure 1. Simplified reaction scheme showing a catalytic mechanism previously proposed for light-driven decarboxylation of fatty acid substrates to form the corresponding alkane catalyzed by fatty acid photodecarboxylase (FAP).[1] The red box indicates the formation and decay of the red-shifted flavin intermediate investigated in the present work.

decarboxylation chemistry to yield an alkyl radical, prior to formation of the final alkane product.[1] However, direct detection of the proposed initial reaction intermediates in support of such a mechanism is still lacking. During the catalytic cycle of FAP, a red-shifted FAD intermediate is formed in approximately 100 ns. On the millisecond time scale, this species decays to the resting FAD (oxidized) species (Figure 1).[1] Similar red-absorbing species have been observed only in the catalytic cycle of a limited number of flavoenzymes, most notably, the flavin-dependent disulfide oxidoreductases such as lipoamide dehydrogenase and glutathione reductase.[16−21] Despite its potential importance in the catalytic mechanism of FAP, the chemical nature of the red-absorbing species is unknown, although it has been proposed to involve the possible deprotonation and reprotonation of a neighboring active site residue or water molecule.[1] Here, through the use of steady-state, laser flash photolysis, cryogenic trapping, and mutagenesis measurements, we spectroscopically characterize the red-shifted flavin species of FAP from *Chlorella variabilis* (*Cv*FAP). Our work illustrates how this intermediate is linked to the protonation state of an active site cysteine (C432, numbering in full-length *Cv*FAP), which is essential for photodecarboxylase activity. On the basis of these findings, we propose a photocatalytic mechanism that involves hydrogen atom transfer from the cysteine to the alkyl radical to yield the final alkane product coupled to the transient formation and decay of a putative FAD C4a-thiolate charge-transfer species.

Initially, the spectral features associated with the red-shifted flavin species were monitored by cryogenic absorbance measurements at 77 K after blue-light illumination for 10 min at a range of successive temperatures. This approach allows reactive intermediates to be cryotrapped and provides additional spectroscopic information by sharpening up many of the flavin absorbance bands (Figure S1, Supporting Information). Absorbance spectra show that an initial light-dependent reaction results in the formation of the red-shifted flavin intermediate, which is characterized by a decrease in the absorbance band at 465 nm and simultaneous increase in absorbance at 406 and 517 nm (Figure 2 and Figure S2). To investigate the apparent temperature dependence of the formation of the red flavin intermediate and to determine the associated energetic/thermal barriers, we measured the rate of increase in absorbance at 517 nm at various temperatures (Figure S3). An activation energy for the formation of the red-shifted FAD species was determined to be 8.1 kJ mol^{-1} (Figure 2A inset) by fitting these data to the Arrhenius function, indicating a relatively weak dependence on temperature. As the red-shifted intermediate can form below 200 K, a temperature that is generally regarded as the "glass transition" of proteins,[22] it is unlikely that large-scale motions play a role in this catalytic step.

To characterize the nonphotochemical reaction steps, we illuminated the samples with blue light (180 K for 60 min) to form the red-shifted state and then warmed them to progressively higher temperatures in the dark for 10 min before recooling to measure absorbance spectra at 77 K (Figure 2C and Figure S4). Difference spectra show that the observed absorbance changes are a mirror image of those measured for the formation of the red-shifted intermediate (Figure S5). Hence, they simply represent the formation of one state that converts back again to the starting state, which suggests that there is a single "dark" step required to recover the initial resting FAD species. The temperature dependence of the nonphotochemical step, obtained by plotting the increase in absorbance at 465 nm (or the decrease at 517 nm) as a function of temperature, shows that it can occur only above 200 K (Figure 2C inset and Figure S6). As this step occurs above the glass transition temperature of the proteins,[22] it is therefore likely to be linked to protein conformational change.

The kinetics of the formation and decay of the red-shifted intermediate were measured by following the absorbance changes at 520 nm upon laser excitation at 470 nm in the presence of excess palmitic acid. Using this approach, we observed kinetic transients on the nanosecond−millisecond time scale, which are similar to those previously recorded by others.[1] An initial increase in absorbance at 520 nm (with an associated lifetime of approximately 180 ns) represents the formation of the red-shifted flavin intermediate (Figure 3A). This is followed by a slower decrease in absorbance (with an associated lifetime of approximately 2.6 ms) as the red-shifted intermediate decays back to the initial FAD species (Figure 3B). Similar kinetic transients were measured over a range of wavelengths and show that the difference spectrum of the species formed in these measurements is almost identical to that observed from the cryotrapping data (Figure S7).

Formation and decay of the red-shifted intermediate could involve H-transfer events.[1] To identify any potential associated solvent isotope effects (SIEs), we performed the above kinetic measurements in buffer solutions containing either H_2O or
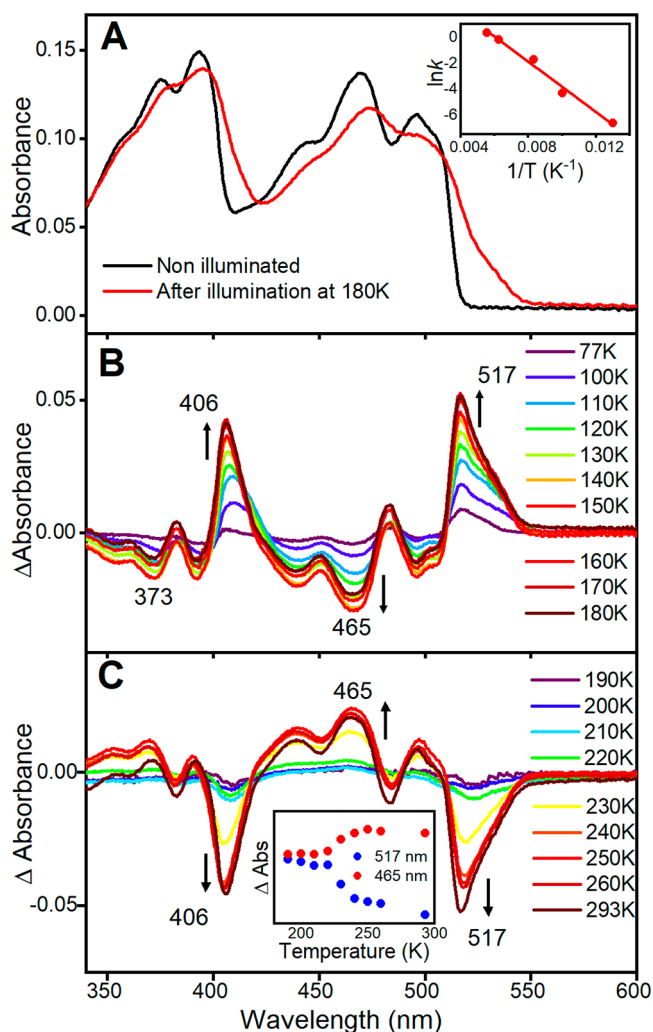
Figure 2. Formation and decay of the red-shifted flavin intermediate monitored by cryogenic absorbance measurements at 77 K. (A) Absorbance spectra before and after illumination for 10 min at 180 K. Inset shows an Arrhenius plot of ln k vs $1/T$ for the rate of formation of the red-shifted FAD species (raw kinetic traces are shown in Figure S3). (B) Difference spectra after successive 10 min illumination at increasing temperatures from 77 to 180 K. The nonilluminated sample was used as a baseline. (C) Difference spectra after illumination at 180 K for 60 min and incubation in the dark for 10 min at increasing temperatures. The inset shows the temperature dependence for the decay of the red-shifted FAD species by plotting the absorbance at 465 or 517 nm. All samples contained 12 μM CvFAP and 250 μM palmitic acid. Formation and disappearance of absorbance features at higher temperatures are indicated by the arrows. Raw absorbance data are shown in the Supporting Information.

Figure 3. Formation and decay of the red-shifted flavin intermediate monitored by time-resolved laser photoexcitation measurements after excitation at 470 nm. Kinetic transients at 520 nm on the microsecond (A) and millisecond (B) time scale of samples containing 40 μM CvFAP and 300 μM palmitic acid in H_2O and D_2O buffer. Data were fitted to a single exponential equation to obtain lifetimes (black lines). (C) Kinetic transients at 560 nm on the millisecond time scale of 25 μM Phenol Red in the presence and absence of 20 μM FAP and 300 μM palmitic acid. Data were fitted to a single exponential equation to obtain lifetimes (black lines). (D) Kinetic transients at 520 nm on the millisecond time scale of samples containing either 40 μM wild-type FAP, C432S variant FAP, or Y466F variant FAP in the presence of 300 μM palmitic acid. All transients were measured at room temperature and data shown are the average of at least five traces.

D_2O. The rate of formation of the red-shifted intermediate is decreased slightly in D_2O (associated lifetime of ~215 ns), resulting in an SIE of ~1.2 (Figure 3A). Although significant, this small SIE suggests that H-transfer events do not have a major effect on the rate of formation of the red-shifted flavin intermediate. On the basis of these data, we infer that the rate of formation of the red-shifted intermediate is mainly limited by other processes (e.g., electron transfer). In contrast, the rate of decay of the red-shifted intermediate in D_2O (lifetime of ~5.35 ms) had a larger SIE of 2.05. Decay of the red-shifted FAD intermediate is, therefore, likely to be associated with or coupled to H-transfer. This was confirmed using laser
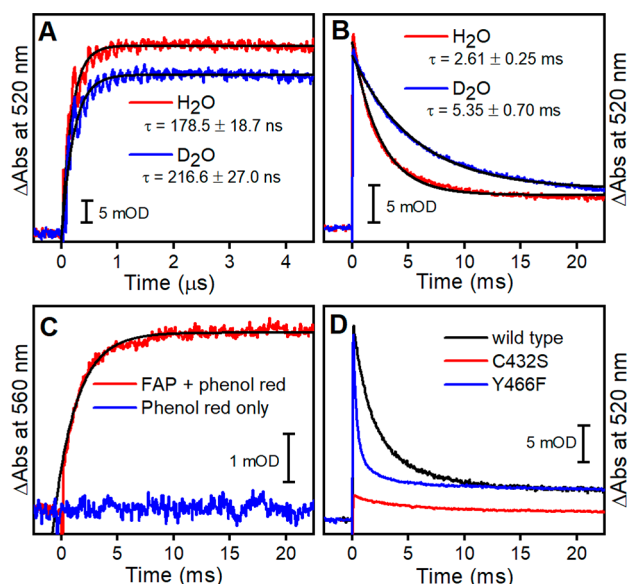
photoexcitation measurements performed in unbuffered solution, using the pH indicator Phenol Red. The observed increase of the Phenol Red absorption at 560 nm was used to monitor the consumption of protons from bulk solvent (Figure S8). Upon laser excitation at 470 nm of CvFAP in the presence of palmitic acid, an increase in absorbance at 560 nm was observed on the same millisecond time scale (lifetime of ~2.05 ms) as the absorption changes attributed to the decay of the red-shifted species (Figure 3C). This time-dependent change in the Phenol Red signal is taken to indicate proton transfer from bulk solvent in this step of the catalytic cycle.

It was previously suggested that the red-shifted flavin intermediate represents formation of a negatively charged amino acid residue positioned close to the FAD cofactor.[1] On the basis of the crystal structure of FAP, the most likely candidates are Cys432 or Tyr466 (numbering in full-length CvFAP). Mutagenesis of both residues to alanine resulted in variant forms of CvFAP that were unable to bind FAD.[1] In the present work, we constructed more conservative mutations (i.e., Cys→Ser and Tyr→Phe), and this resulted in FAP variants that retained the FAD cofactor (Figure S9). Steady-state analysis showed that the Y466F variant retained catalytic activity, whereas the C432S variant was inactive (Figure S10), implying that Cys432 is important for photocatalysis. Moreover, kinetic measurements demonstrated that the C432S variant does not form the red-shifted flavin intermediate (Figure 3D). This implies that Cys432, located approximately 5 Å from the carboxyl group of the palmitate substrate (Figure

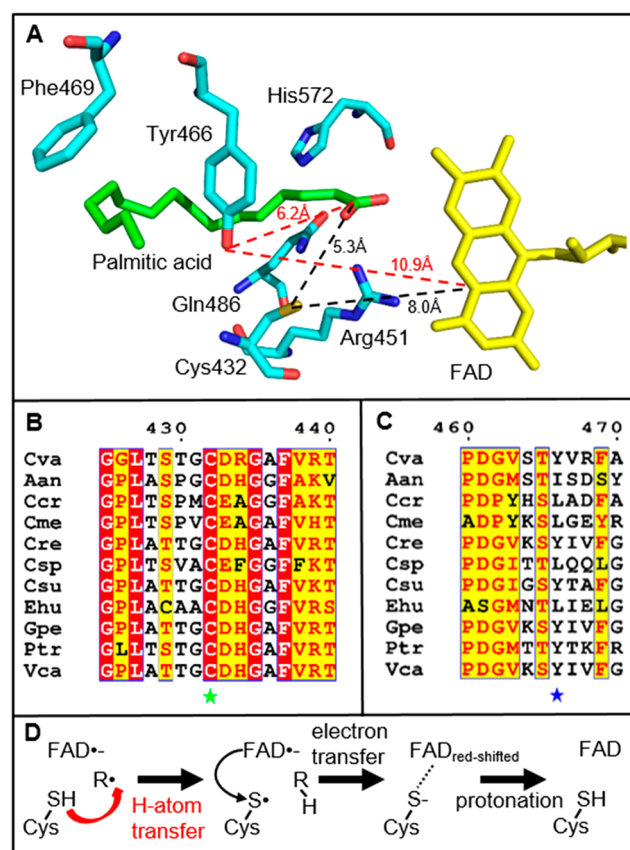4A), is the most likely candidate residue involved in the formation and decay of the red-shifted intermediate.

Figure 4. Proposed role of Cys432 in the reaction mechanism of FAP. (A) Structure of the active site of *Cv*FAP, based on the crystal structure of the enzyme.[1] The FAD cofactor is shown in yellow, palmitic acid substrate is shown in green, and active site residues are shown in cyan. Distances from the SH group of Cys432 (black dashed line) and phenolic hydroxyl group of Tyr466 (red dashed line) to the C4a of FAD and carboxyl group of palmitic acid are shown. (B) and (C) are the sequence alignment of selected regions for multiple FAP enzymes using T-coffee multiple sequence alignment[25] and ESPript software.[26] The highly conserved Cys432 is highlighted with a green asterisk (B) and Tyr466 is highlighted with a blue asterisk. (D) Schematic showing the proposed mechanism for the formation of the flavin thiolate charge-transfer species via concerted or sequential H atom and electron-transfer reactions.

Conversely, the Y466F variant does form the red-shifted species with an absorption intensity similar to that of the wild-type enzyme (Figure 3D). However, the subsequent reprotonation step (millisecond time scale) is faster in this variant (Figure 3D). This might reflect differences in local reorganization in the active site (e.g., water networks, which are implicated in reprotonation of Cys432).

Taken together, these studies support the involvement of a red-shifted flavin intermediate in FAP photocatalysis. This intermediate is likely to involve an interaction between the thiolate of Cys432 with the C4a atom of the FAD isoalloxazine ring to form a FAD C4a-Cys432 charge-transfer species. This is not unprecedented as similar species are well-known in the flavoprotein disulfide oxidoreductase family of enzymes, such as lipoamide dehydrogenase and glutathione reductase.[16−21] Moreover, a covalent adduct between an active site Cys residue

and the C4a of FMN is also formed in the photocycle of the photoactive LOV domains, although this species has a significantly blue-shifted absorbance maximum that is likely to be due to a protonated flavin N5.[23,24] Although a similar covalent adduct is unlikely in *Cv*FAP based on the distance (∼8 Å) between the thiolate and the flavin C4a in the crystal structure[1] (Figure 4A), it is possible that localized protein/cofactor motions could enable formation of a FAD C4a-Cys432 charge-transfer intermediate. Such an electron donor−acceptor complex would be stabilized by π-stacking interactions between the electron-rich sulfur and the electron-deficient FAD cofactor. Cys432 is also strictly conserved in all FAP sequences (Figure 4B), whereas Tyr466 (*Cv*FAP) is less well conserved in other FAP sequences (Figure 4C). This further highlights the inferred importance of Cys432 to the photocatalytic cycle, suggesting the red-shifted flavin thiolate charge-transfer species may be common to all FAP enzymes.

On the basis of the present work, we propose a mechanism for the formation of the red-shifted flavin intermediate, which occurs after the excited-state ultrafast decarboxylation chemistry (Figure 4D). It is likely that the deprotonated state of the Cys residue in FAP arises from an initial H atom transfer to the decarboxylated alkyl radical to yield a Cys thiyl radical and the final alkane product. The Cys432 thiol group is sufficiently close to the alky radical (∼5 Å in the published crystal structure) for this to occur. The Cys thiyl radical would then be quenched by electron transfer from the FAD semiquinone to Cys432 to form the thiolate species. This could occur in a concerted or sequential manner; regardless, both steps are complete in ∼170 ns. The thiolate is subsequently reprotonated on the millisecond time scale to initiate a new photocatalytic cycle.

In conclusion, on the basis of mutagenesis, laser spectroscopy, solvent isotope effects, and cryotrapping studies, we have highlighted the importance of a strictly conserved active site Cys residue in the catalytic cycle of FAP enzymes. We propose that this residue forms an FAD C4a−Cys thiolate charge-transfer species in the light-dependent decarboxylase mechanism, thereby accounting for the transient "red intermediate" observed in the natural photocyle of the enzyme. These studies provide important, new information on how light energy can be harnessed to drive flavin-based decarboxylation chemistry. Our work identifies a general chemical strategy in which light-activated decarboxylation is closely coupled to H-transfer chemistry mediated by a strictly conserved Cys residue in the active site of FAP enzymes. Mechanistic insights emerging from this study will have general implications for understanding the photocycles of flavin-dependent enzymes and more generally will inform on the design of novel flavin-dependent photocatalysts.

■ ASSOCIATED CONTENT

ⓈI Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.0c01684.

Details of all experimental methods; raw data shown in Figures S1−S10 (PDF)

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Author Contributions

D.J.H., B.L., S.J.O.H., M.S., and T.M.H. performed experiments and analyzed data. D.J.H. and N.S.S. initiated and coordinated the research program. D.J.H. wrote the manuscript through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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